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DNA Methylation and Gene Expression

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INTRODUCTION

Although the presence of 5-methylcytosine in animal cell DNA was noted over 40 years ago, its role in the regulation of gene activity has become apparent only recently (8, 12, 13, 62). The first indications that DNA methylation patterns of particular genes may indeed be related to their expression profile were revealed by restriction enzyme and blot hybridization analysis of genomic DNA from different tissues. The first experiments were done on the rabbit (84) and chicken globin (49) genes and later on the human globin gene (81) by using the enzymes *Hpa*II (CCGG) and *Hha*I (GCGC), which are inhibited by methylation at cytosine residues in their recognition sites. These studies showed clearly that the globin gene was unmethylated in the tissue of expression but heavily modified in DNA from other tissues. Similar results were obtained subsequently for many other gene sequences in a variety of organisms (96). Thus, for tissue-specific genes, there is a fairly straightforward correlation between expression and undermethylation. In keeping with this structure, housekeeping genes all contain a CpG-rich island region at their 5′ end, and, when active, these islands are completely unmethylated in all tissues (9, 73).

TRANSFECTION EXPERIMENTS

Although studies of gene specific methylation patterns suggested that DNA modification may be involved in gene regulation, they did not prove that methylation of a gene is the direct cause of its suppression. To evaluate this question, it was imperative to turn to tissue culture cells as a model system. By and large, these cells are quite similar to their in vivo counterparts in that most genes have the same methylation pattern as in authentic tissues and these modifications tend to be fairly stable even after many generations of growth in culture (96). Thus, these cells must have a mechanism for carrying on a particular methylation pattern from generation to generation, and this has been confirmed by transfecting foreign DNA into tissue culture cells (72, 88). When DNA is introduced in its unmethylated form, it retains this unmodified pattern continuously. On the other hand, in vitro methylated constructs remain modified, suggesting that these fibroblast cells have neither demethylation nor de novo methylation activities and are strictly capable of maintaining a given pattern. In vivo, methyl moieties are found almost exclusively at CpG residues, and when the cell is presented with a template that is modified at every cytosine, it will maintain methyl groups only at these sites (72). It is likely that the dyad symmetry of the CpG dinucleotide is what makes it a preferred substrate for methylation. In vivo, each site is actually modified on both strands of the DNA (7, 14). Immediately following replication, however, the newly synthesized strand is, as yet, unmodified and maintenance is performed by an enzyme which is highly specific for hemimethylated residues (23). Thus, any CpG site which is initially unmodified will remain that way after replication, while a methylated CpG site will be recognized by the methyl group remaining on the parental DNA and will thus be remodified in the complementary strand. The importance of cytosine symmetry in this mechanism is indicated by the fact that non-CpG methyl moieties in plant cells are all found in the trinucleotide symmetrical sequence CXG (24).

DNA-mediated gene transfer can also be used to evaluate the effect of methylation on gene expression. When the tissue-specific human γ- or β-globin genes were methylated in vitro and transfected into fibroblast cells, it was clearly shown that DNA modification suppresses transcription; this indirectly suggests that the endogenous globin genes in the same cell are also inactive because of their modification state (11, 95). The effect of methylation in this assay appears to have a broad spectrum of activity, since almost all transfected genes are repressed when modified and since the same phenomenon can be observed in a variety of tissue culture cell types and in *Xenopus* oocytes (19, 82). This effect is also not limited to in vitro-methylated templates. Both the inactive X-linked *Hprt* gene (46) and a modified muscle determination gene in 10T1/2 cells (45) have been shown to be inactive in the transfection assay, and the same can be shown for the transcriptionally silent mouse endogenous viral sequences (74).

5-AZACYTIDINE EXPERIMENTS

Further evidence indicating that DNA methylation inhibits gene expression has been obtained with the drug 5-azacytidine (5-azaC), a potent demethylating agent (32). This com-

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pound, which is a cytosine analog, incorporates into DNA and probably binds the maintenance methylase in an irreversible manner, thus sequestering the enzyme and preventing maintenance of the proper methylation state (33). One generation in the presence of the drug is sufficient to cause much of the DNA to become hemimethylated, whereas double-strand demodification can be observed after the next division. When certain cells are treated in this way, selective gene activation can be observed. For example, the inactive endogenous virus gene in chicken AEV cells was turned on after the cells were exposed to 5-azaC, and the gene sequences were found to have undergone demethylation (22). Similarly, other individual genes undergo activation in different cell types. Many genes have been shown to be in a hemizygous state in cultured CHO cells. The inactivation of only one allele in these cells presumably involves DNA methylation, since 5-azaC can activate them at high efficiency (25). A similar phenomenon takes place for genes on the inactive X chromosome, and this can best be seen in the experimental $Hprt^-$ cell lines in which the gene on the active X chromosome is mutated while the normal copy is on the inactive chromosome (51). For some cell lines of this nature, treatment with 5-azaC converts over 10% of the cells to the $hprt^+$ phenotype and allows it to become transfectable (83). In this system 5-azaC is not merely turning on individual methylated genes, since the same cells show high-frequency activation of other X-linked genes (57) and one can even observe conversion of the X chromosome to an early replication time under transient-transfection conditions (29). The timing of satellite DNA replication is also affected by 5-azaC treatment (67).

The activation potential of 5-azaC is best seen in 10T1/2 or NIH 3T3 cells, in which treatment causes differentiation to three different distinct mesodermal cell types (78). A careful analysis of the range of proteins which become expressed following induction showed that demethylation at each individual locus could not explain the frequency of this differentiation process. It was therefore hypothesized that 5-azaC works at a few discrete loci, perhaps in a small number of master genes, which then go on to direct the developmental program by turning on other tissue-specific genes in a sequential manner (41). In fact, one gene of this nature, *myoD*, has already been cloned and shown to have the ability to initiate differentiation to myoblasts in certain cell types (15). Despite the fact that this gene is methylated in 10T1/2 cells and undermethylated in the myoblast (34), it is still not clear which gene is the initial target for 5-azaC action. In fact, there is at present no hard evidence indicating that this drug exerts its effect through demethylation, since it is also known to have other effects on the cell, including the ability to cause chromosomal aberrations (32). It is interesting that many of the genes which are activated by 5-azaC are actually in a nonphysiologic state of methylation to begin with. This is certainly the case for MyoD, which contains a CpG island that is normally unmethylated in all tissues of the organism but is anomalously heavily modified in 10T1/2 cells prior to its induction by 5-azaC (34).

GENE REPRESSION BY METHYLATION

The details of how DNA methylation affects gene transcription are slowly being resolved, but it is clear that the basic mechanism works by altering protein-DNA interactions. The effects of modification in vivo probably involve many different proteins, including those which go into forming the chromatin structure. When DNA is introduced into cells by DNA-mediated gene transfer, it integrates into the genome and is always found in a DNase I-sensitive chromatin conformation (37). In sharp contrast, methylated DNA is put into an insensitive structure, and this clearly suggests that the methyl moieties have a role in the placement of proteins required for building the correct conformation. Since this phenomenon occurs for any DNA, the process probably does not involve sequence specificity. Additional evidence for this type of global mechanism was also seen in transiently transfected cells, in which methyl moieties appear to inhibit transcription, but only after enough time has passed for the chromatin structure to form on the vector DNA (10). Although the exact details of this mechanism are not known, recent studies have identified a unique protein which specifically binds to methylated residues and which presumably has a dramatic effect on overall chromatin structure (50).

Methyl moieties may also interfere with the binding of individual proteins to DNA, and this has been demonstrated nicely for factors which interact with the liver-specific *tat* gene (3). In this case, in vivo footprinting indicated the presence of several tissue-specific loci of protein binding upstream to the gene. When tested in vitro on plasmid DNA, however, it became clear that these footprints were actually made by ubiquitous factors found in all cell extracts. Thus, in vivo, the lack of apparent factor binding must be due to differential methylation patterns between the liver and other tissues. This idea was easily confirmed by showing that in vitro DNA methylation actually inhibits the binding of these factors. More recently, other workers have also shown that methylation at specific sites can interfere with the binding of protein factors, including some known to be required for RNA synthesis (28, 85). An exception to this is Sp1, which binds and activates even when the recognition site is modified (26). This type of effect also explains why, for many genes, methylation in the 5′ regulatory region is sufficient for inhibition of gene activity (11, 38, 43, 95). It should be noted that it has been difficult to demonstrate an effect of DNA modification in animal cells by using in vitro transcription on naked DNA (16), and this indirectly implicates overall chromatin structure as an important element in the inhibition mechanism. In plants, on the other hand, a few genes are strongly inhibited by methylation in an in vitro assay system, suggesting that in these cells methyl moieties may directly interfere with the action of transcription factors (39).

DNA METHYLATION AND DEVELOPMENT

Although it is quite clear from experiments in tissue culture that DNA methylation inhibits gene expression, it is not obvious how this mechanism might operate in vivo. To understand the role of modification during development, it is necessary to monitor the fate of DNA methyl moieties as a function of cell differentiation. All tissue-specific genes that have been analyzed are methylated in sperm DNA. The methylation state of these same genes in oocyte DNA is not known as yet, but at some early stage in embryonic development tissue-specific genes are methylated in almost all tissues. Only in the tissue of expression does a particular gene undergo demethylation, and this occurs at approximately the same time that it becomes actively transcribed (5). It is clear that understanding of the complete picture will require more information, especially on the patterns of methylation at very early stages of embryogenesis and gametogenesis (13).

In addition to demethylation, some genes may undergo de

novo methylation at certain stages of development. The best examples of this phenomenon are the housekeeping genes on the X chromosome, which become selectively modified on the inactivated chromosome at some time after the blastocyst stage (47, 71). It is this new methylation pattern which then maintains the X chromosome in the inactive state in all cells for the lifetime of the organism. In overview, it appears that most tissue-specific genes are methylated at least at later stages of development and in a large variety of cell types. Methylation thus serves as a general signal for inactivation, which does not require cell-specific or sequence-specific repressors. This principle is best observed for X-chromosome genes in female cells. Since the signal for repression is forged into the DNA sequence itself, both the active and inactive gene can coexist in one cell despite being exposed to the same transcription factors.

DEMETHYLATION IN SPECIFIC CELL TYPES

According to the above picture of in vivo methylation dynamics, one would expect that during development, specific cell types must have the potential to recognize certain genes in their modified state and thus change their status through both demethylation and transcriptional activation. By using tissue culture cell lines, it was possible to show that this is indeed the case. In most transfection studies, methylated sequences were inserted into fibroblast cell lines, which would not be expected to have a mechanism for recognizing these specific genes. In contrast, when the muscle-specific α-actin gene was inserted into the L8 myoblast cell line, unique CpG sites in the upstream regulatory region underwent striking demethylation; this was correlated with gene activation (94). As expected, this effect is sequence specific, and other, nonmuscle genes do not become demethylated in L8 cells. Similar results have been obtained for the rat insulin gene in an insulinoma cell line and for immunoglobulin κ chains in mouse lymphocytes (20). These studies suggest that at least some differentiated cell types retain the ability to recognize the appropriate genes and carry out their demodification in a manner similar to that which occurs during normal development in vivo.

Although both demethylation and gene activation occur following stable transfection to specific cell types, it is impossible to evaluate the temporal relationship between these two events, since analysis can be done only after the cells have gone through a large number of generations. To address this question, L8 myoblasts were transiently transfected with the α-actin gene. Under these conditions it was possible to show that demethylation on one strand of a particular CpG site occurs within a few hours after the introduction of the template into the cell, while full demodification is attained only at later times (48 to 96 h) (56). In this experiment, the α-actin gene became active only at the later times, when both methyl groups were removed from the critical sites. This result was also confirmed by transfecting a modified mutant gene which has all of the sequences required for transcription, but is unable to undergo demethylation. In this case, active transcription was severly inhibited in L8 cells even following long-term stable transfections.

Although this represents one example of gene activation during development, there is no reason to believe that all tissue-specific sequences behave in a similar manner. Indeed, several liver-specific genes appear to undergo demethylation following their activation in the fetal liver. An instructive example of this phenomenon is provided by rat *PEPCK* (5). This gene becomes fully expressed at birth, yet many CpG sites in the gene domain are still fully methylated, and the methyl groups are removed from the sequence a few days later. A similar phenomenon is observed with the rat albumin (44) and chicken vitellogenin (89) genes. In the latter case, methylation at every CpG residue was determined by genomic sequencing, and in this manner it was shown that, at the initiation of transcription, 11 CpG sites are already fully demethylated but 4 sites are still hemimethylated (65). These data suggest that these genes may be activated by factors which override the effects of methylation. Following this initial event, the gene undergoes demethylation, which now allows transcription in the absence of the initial differentiation factors. This is exactly what is seen in mouse pre-B cells. When treated with lipopolysaccharide, the κ gene, which has already rearranged, is transcriptionally activated but remains completely methylated (53). Only following differentiation to mature B cells does this gene undergo demethylation (36). Further studies are required to verify this mechanism, since in none of these cases has it been shown that methylation at these sites is involved in gene repression.

It is quite likely that overriding of methylation does play a role in the transcription of several viral sequences. Frog virus, for example, is completely methylated at every cytosine residue, and this situation is sufficient to inhibit expression of individual genes. This virus, however, contains a factor which is capable of enabling transcription, and this allows the virus to propagate in animal cells despite its state of modification (79, 90). Both adenovirus (86) and human immunodeficiency virus (4) also appear to be transcribed despite the presence of methyl moieties which are known to inhibit RNA synthesis. In the latter case, it is thought that the *tat* gene is required for this overriding effect (4).

MECHANISM OF DEMETHYLATION

It has always been assumed that demethylation in vivo takes place through a passive mechanism whereby the maintenance methylase is inhibited at specific sites. In this scenario, full double-stranded demethylation would require at least two cell divisions and would occur in only 50% of the cells (62). Although this type of demodification does take place following treatment of cells with 5-azaC (78), there is, as yet, no evidence that this is the mechanism in vivo. Indeed, in several instances it has been proven that demethylation takes place through an active mechanism. Mouse erythroleukemia cells, for example, undergo a genome-wide transient demethylation in response to treatment by hexamethylene bisacetamide. By carefully monitoring the kinetics of this reaction, it was shown that methyl groups are removed at times in the cell cycle when DNA is not undergoing replication. In fact, double-strand demodification can be observed after a few hours of treatment, much earlier than the time required for two cycles of replication (61). A similar phenomenon is observed for Epstein-Barr virus, which undergoes extensive and rapid demethylation in Burkitt's lymphoma cells (76). This effect has also been confirmed in other systems in vivo. Both δ-crystallin (75) and vitellogenin (65) become demethylated in their specific tissues by an active mechanism since methyl removal occurs even when DNA synthesis is inhibited.

Perhaps the best evidence for an active mechanism comes from studies of the α-actin gene transiently transfected into L8 myoblasts. In this system demethylation occurs on unintegrated plasmid molecules. Since these are derived

from bacteria, they also carry a methylated adenine at all GATC sites. By using the restriction enzymes *Dpn*I and *Mbo*I, it could be shown that during the process of demethylation these molecules do not undergo any replication, as evidenced by the persistence of 6mA residues. These experiments showed clearly that molecules which underwent demethylation did not undergo replication. Further support for an active mechanism was obtained by demonstrating that the demethylation actually occurred on prelabeled DNA strands. This would have been impossible in a passive mechanism (56).

Very little is known about the actual biochemical mechanism of demethylation. Experiments in erythroleukemia cells suggest that this occurs through replacement of 5-methylcytosine with cytosine (63). This reaction would be similar to that of glycosylases which are known to be involved in the removal of uracil or aberrant bases from DNA. The recent demonstration of enzymatic correction of T-G mismatches in an in vitro reaction (87) gives some hope that demethylation can be studied by similar means. Only then will the exact reaction mechanism be amenable to analysis.

It is clear that demethylation in vivo may represent an early event in the multistep process leading to the recognition and activation of *cis*-acting sequences during development. Elements required for the demethylation reaction of α-actin have already been found in the regulatory region of this gene (56). With this preliminary information in hand, it should now be possible to fully characterize the nature of these sequences and to identify this new category of *trans*-acting factors which carry out the recognition process and are obviously necessary for early stages in cell differentiation.

DYNAMIC CHANGES OF DNA METHYLATION IN EMBRYONIC CELLS

The picture of DNA methylation which emerges from studies with somatic cells suggests that modification patterns are fairly stable. Expressed alleles of housekeeping genes have CpG islands at their 5′ end which are perpetually unmethylated in all cells of the organism (9). Tissue-specific genes, on the other hand, are generally methylated in all somatic cells (96). Only in the specific cell types of expression does a gene undergo demethylation, and, following this event, the new pattern is faithfully maintained in this cell type and remains that way even in tissue culture (60, 88).

Little is known about the state of DNA methylation in the embryo germ line axis. Although initial studies suggested that the level of methylation in the embryo is similar to that in somatic cells (70), more recent findings indicate that these early stages are actually characterized by waves of massive alterations in the modification patterns of the genome. Monk et al. (52), using a highly sensitive but nonspecific assay for DNA methylation, monitored the overall level of modification throughout embryogenesis. Their findings suggest that methylation levels are very high in sperm DNA, but much lower in oogonia, and that in early morula stages of development the degree of modification represents an average of the maternal and paternal genomes. This pattern has also been confirmed for several specific multicopy genomic sequences (66). According to these data (52), a further demethylation event may occur at the blastocyst stage and is followed by a wave of de novo methylation which takes place around the time of implantation. These authors also suggested that germ line lineages escape the de novo reaction; this is supported by experiments showing that several tissue-specific genes are indeed unmethylated at early oogonial stages in human embryos (17).

Although this model has not been verified experimentally for individual genes, results of additional studies indeed support portions of this scheme, and in particular the de novo methylation which takes place prior to day 7.5 in the mouse. The existence of this reaction can easily be observed when embryos are infected with murine leukemia virus before implantation, since this virus then undergoes inactivation and de novo methylation. In contrast, murine leukemia virus is permissive when infection is carried out after implantation, and in this case the integrated viral sequences remain unmethylated (31). The same reaction can also be observed in tissue culture cells, in which murine leukemia virus becomes methylated in rapidly dividing embryonic carcinoma cells, but not in the same cells after they have undergone differentiation as a result of retinoic acid treatment (30).

De novo methylation of other gene sequences can be observed in transgenic mice (20, 68). The kinetics of this reaction have not yet been studied, but in every case that has been investigated, tissue-specific transgenes appear to have undergone de novo methylation, even in the founder mice. In contrast, island sequences are protected from this process and thus remain unmethylated (40, 68). Further insight into the mechanism of this selectivity was obtained by making a transgenic mouse by using a methylated *APRT* gene (20). In this case, the 5′ CpG island actually underwent demethylation in founder mice, whereas the nonisland portions of the gene remained methylated and even became de novo methylated at sites not originally modified in vitro. These experiments suggest the presence of an island demethylation activity in embryonic cells, and this was confirmed in teratocarcinoma cells growing in culture. In these cells, as opposed to somatic cell types, a transfected *APRT* gene is adjusted in vivo to imprint the correct methylation pattern; this is done by means of a combination of demethylation and de novo methylation activities in the cell. Since this also appears to occur in vivo, these studies imply that the early embryo has the capability to recognize classes of gene sequences and reestablish their correct methylation pattern, which is then carried on to somatic cells, where it is stably maintained. By using transfection into embryonic cells in culture, it should now be possible to evaluate the *cis*-acting sequences and *trans*-acting factors involved in recognizing and properly modifying gene sequences (77). In addition, an animal cell DNA methyltransferase has recently been cloned, and this should be useful for analyzing how methylation patterns are established (6).

De novo modification is also associated with the X-chromosome inactivation that occurs in female eutherians in vivo (32). In somatic female cells, genes on the inactive X chromosome are transcriptionally silent, and several studies have confirmed that these inactive housekeeping genes are highly methylated in their CpG islands in comparison with the same gene on the active X chromosome. This has been best demonstrated for the mouse *Pgk-1* gene, in which 120 CpG residues in its CpG island were found to be completely methylated in the inactive X chromosome and totally unmodified when the chromosome was active (59). The process of X-chromosome inactivation is initiated in the inner cell mass in the late blastocyst, but de novo methylation occurs after this event and some modifications may actually take place a few days following inactivation (47, 71). For this reason it has often been pointed out that DNA methylation of the X chromosome acts as a maintenance mechanism and

is not the initiator of inactivation. This is supported by the fact that X-chromosome inactivation in extraembryonic tissues occurs without subsequent modification (42). In general, de novo methylation is probably a secondary event, which usually takes place following a decision to inactivate, as is the case for murine leukemia virus inserted into F9 teratocarcinoma cells (21). The unusual stability of X-chromosome inactivation is probably directly attributable to the methylated state and the inability of the CpG island containing genes to undergo demethylation in somatic cells. This conclusion is supported by several key experimental observations. Most important is the fact that activation of some genes on the inactive X chromosome can be demonstrated following treatment with 5-azaC (51). Furthermore, in marsupials, in which DNA methylation does not accompany the X-chromosome inactivation process, spontaneous age-dependent reactivation indeed occurs at a high frequency (35).

DNA METHYLATION PATTERNS IN CELL LINES

Analysis of endogenous genes and of transfected sequences in somatic cells in culture suggests that DNA methylation patterns are stable in such lines. Despite this generalization, alterations in DNA methylation can also be observed in culture, although in most cases, this process is slow and may occur over many generations. When tissue cells are first put into culture, there is evidently a rapid general loss of methyl moieties, but as immortal cells emerge, the methylated-DNA content appears to increase (48, 91). An example of this process at the level of a single gene has been documented for MyoD (34). This gene has a large intronic CpG island, which is fully unmethylated in all tissues of the mouse. In 10T1/2 and other fibroblastlike cells, however, this island is modified, and it has been suggested that this is one of the target genes for the 5-azaC-induced differentiation to myoblasts in culture. By monitoring the methylation pattern of this gene during the establishment of a cell line, it was shown that the sequence undergoes a progressive process of de novo methylation, culminating in the emergence of the immortal line. Since activation of MyoD at any time during these passages could presumably lead to differentiation and a cessation of cell division, it has been proposed that this system actually selects for cells in which the CpG island has undergone fortuitous de novo methylation. If the methylated state of this gene does indeed grant the cell a growth advantage, cells containing a modified sequence would eventually overgrow the culture.

Further evidence that this type of process occurs during cell immortalization comes from studies by Antequera et al. (2), in which a large number of CpG island sequences were examined. Although island DNA is generally unmethylated in all tissues of the intact organism, it was found that CpG islands of many nonessential genes are highly methylated in tissue culture cells. These changes also presumably occur over a long time scale under growth-selective conditions (69, 92). The same type of methylation may also be responsible for the observed modification of exogenously introduced viral sequences several generations after their transfection and of genes which have undergone repression or extinction in certain cell lines (1, 27, 54). Changes in the methylation patterns of specific genes are also observed in tumor cells (64). This, too, may involve selective pressure, since in at least one case, the degree of demethylation appears to be correlated with the extent of metastatic potential (18).

Another phenomenon seen in tissue culture cells is methylation variability at certain sites. This is usually characterized by a stable state of incomplete modification at a specific CpG residue (93). When subclones are prepared from individual cells, each new colony is also partially methylated. It has been suggested that a balance between the rates of de novo methylation and maintenance methylation is involved in maintaining this partially methylated state (55, 58). A similar mechanism may be involved in continually maintaining hemimethylated CpG loci in growing cells (80).

CONCLUSIONS

The role of DNA methylation as a locking mechanism for gene expression in somatic cells is well established, but much remains to be done to improve our understanding of the dynamic changes which occur during development. Recent experiments suggest that tissue-specific tissue culture cells maintain their ability to carry out the demethylation of active genes, and this system should now allow the dissection of both the *cis*- and *trans*-acting elements required for the reaction. Since demethylation may precede gene expression during the differentiation process, this could uncover a wide range of new factors which are involved in the earliest stages of sequence recognition and activation. Furthermore, the availability of large amounts of cell material could pave the way for carrying out this reaction in vitro. In that way, it will then be possible to understand the exact biochemical mechanism of demethylation.

Another major area of research involves the changes which occur in DNA methylation during early embryogenesis and in the germ line axis. It will be necessary to use exquisitely sensitive methods to assay DNA methylation at specific sites in oocytes, the morula, and the blastocyst, in which the number of cells is limiting. Preliminary results obtained by using the polymerase chain reaction already suggest that this is possible. In any event, it is quite clear that embryonic cells differ from somatic cells in their ability to restructure methylation patterns, and this process can be studied in vitro by using embryonal carcinoma or ES cell lines. This system should provide the means of dissecting out the *cis*-acting sequences which recognize certain classes of genes and orchestrate the complicated pattern of demethylation and de novo methylation which takes place during this stage. It should also help clarify the role of DNA modification in genomic imprinting.

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